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PREPARATION OF SEVERAL TYPES OF RPC-5-LIKE RESINS AND THEIR USE FOR THE SEPARATION OF OLIGONUCLEOTIDES AND MONONUCLEOTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Improved alternative packing materials to RPC-5 were prepared from granular polychlorotrifluoroethylene of several different particle sizes and trioctylmethylammonium chloride, and used for the separation of mononucleotides and oligonucleotides in high-performance liquid chromatography. High resolution was obtained when particles of 4–8 μm diameter were used, whereas coarser materials (40–90 or 10–40 μm) gave poor resolution. The greater the amount of the quaternary ammonium salt coated, the smaller was the elution volume for the same sample. Oligonucleotides up to a chain length of 75 could be successively separated in less than 2 h under the optimum conditions. Resolution of mononucleotides and short-chain oligonucleotides was accomplished with the resin coated with a minimum amount of the quaternary ammonium salt. A variety of oligonucleotides could be separated depending on the chain length, the differences in the internucleotide linkage and the differences in the base sequence.

INTRODUCTION

The efficient separation of DNA fragments, oligonucleotides and mononucleotides is very important in nucleic aid chemistry and genetic engineering. Up to now the separation and analysis of oligonucleotides have generally been accomplished by high-performance liquid chromatography (HPLC) on a reversed-phase column (ODS-silica gel) or a silica gel-based anion-exchange column[1]. However, these columns cannot be used under alkaline conditions, and are generally inadequate for the separation of long oligonucleotides. Several polymer-based gels have been developed for the separation of oligonucleotides and nucleic acids to overcome the drawbacks of silica gel-based packing materials. For example, Kato and co-workers reported a bonded anion-exchange column based on polymer gel (TSK gel DEAE-5PW) for the separation of oligodeoxyribonucleotides[2] or ribosomal RNA[3].

RPC-5 column chromatography has proved useful for the separation of t-RNA [4–6], DNA restriction fragments[7–9], plasmid DNA[8,10] phage RNA[11] and long oligonucleotides[7,12], since the introduction of RPC-5 by Pearson *et al.*[4]. RPC-5 gives

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a high resolution of long-chain oligonucleotides; it is mechanically strong and chemically inert under strongly acidic and alkaline conditions. However, the original RPC-5 material requires a long time for separations and was not commercially available in high quality, although some alternative materials have been devised. Usher[13] used Kel-F powder as a support for RPC-5 and separated oligouridylates and oligoadenylates. We have now prepared RPC-5-like resins from polychlorotrifluoroethylene with several different particle sizes by modifying the coating and packing methods and have investigated the separation of long- and short-chain oligonucleotides and mononucleotides. The influence of the particle size and amount of coating of trioctylmethylammonium chloride on the resolution and retention volume was studied. This paper reports the usefulness of these RPC-5-like resins for the separation of short-chain oligonucleotides and mononucleotides.

EXPERIMENTAL

Materials

All chemicals were of analytical reagent grade. Buffer solutions were prepared from distilled water and filtered through a 0.22-μm membrane filter.

Oligonucleotides

3′–5′-Linked oligoadenylates containing 5′-terminal phosphate were prepared by partial digestion of polyadenylic acid (Poly A) with nuclease SW[14]. The enzyme digestion was carried out in a solution (1.5 ml) containing Poly A (10 mg), 0.05 *M* carbonate buffer (pH 10.3), 0.1 *M* sodium chloride, 1.5 m*M* magnesium chloride and nuclease SW (100 units) at 37°C. Aliquots of 200 μl were taken after 30 min and 1, 2 and 4 h and stored in a freezer until analysis by HPLC. The chain length of the resulting oligoadenylates depended on the reaction time. Linkage isomers of oligoadenylates and oligouridylates were prepared by lead ion-catalysed polymerization of adenosine-[15] and uridine-5′-phosphorimidazolide[16], respectively. Synthetic oligodeoxyribonucleotides, which were prepared by the phosphotriester method, were a gift from Professor Hata of Tokyo Institute of Technology.

Packing materials

Coarse and fine granular polychlorotrifluoroethyelene (Neosorb ND) were supplied by Nishio Kogyo. Coarse materials were dry-sieved through 100-, 200- and 400-mesh stainless-steel sieves successively to fractionate them into three portions, 100–200 mesh (IV), 200–400 mesh (III) and 400+-mesh (II) particles. A 100-g amount of each fraction was suspended in 200 ml of dichloromethane in a measuring cylinder for sedimentation. The contaminated fine particles in the fraction, which settled slowly, were discarded by suction. Fine materials (I) with a small and uniform particle size had an average particle size of 5–6 μm. The particle size distributions of the materials I, II, III and IV were analysed by the balance sedimentation method in a suspension of isobutyl alcohol. The analyses were carried out by Sumika Analytical Center. The polychlorotrifluoroethylene particles were non-porous, with a nearly spherical to irregular shape with a rough surface which was observed with a microscope. The surface area of material I was 2.47 m^2/g, as measured by the fluidized BET method with a Shimazu Flowsorb 2300 apparatus (Shimazu Techno Research). Table I lists the particle-size distribution of materials I, II, III and IV.

TABLE I

PARTICLE SIZE DISTRIBUTIONS OF POLYCHLOROTRIFLUOROETHYLENE POWDERS

Size range (μm)	*Mass-%*			
	I	*II*	*III*	*IV*
1–2	3			
2–4	36			
4–8	56			
8–10	5			
10–20	0	9		
20–30		36	5	
30–40		55	71	9
40–50		0	18	17
50–60			6	24
60–80			0	29
80–100				21

Coating of Neosorb ND with trioctylmethylammonium chloride was carried out by a modification of the method described by Pearson *et al.*[4]. The original RPC-5 contains 4.0 ml of trioctylmethylammonium chloride per 100 g of Plaskon (polychlorotrifluoroethylene) powder. In our experiments, 100 g of material I were coated with 12, 6 and 3.8 ml of trioctylmethylammonium chloride to prepare RPC-5-like materials Ib, Ia and Ic, respectively. The materials II, III and IV were coated with 6 ml of trioctylmethylammonium chloride.

A slurry of the coated materials in 0.5 *M* sodium perchlorate containing 10 m*M* Tris–acetate (pH 7.5) and 1 m*M* EDTA was packed into a stainless-steel column (25 cm × 4 mm I.D.) with a magnetically stirred slurry packer at a flow-rate of 5.0 ml/min. The pressure rose during packing to 250 kg/cm^2 for the materials prepared from I. The flow was continued for 1 h at a constant pressure of 250 kg/cm^2 to obtain cross packings. The column for the separation of mononucleotides was prepared by coating the polychlorotrifluoroethylene powder with trioctylmethylammonium chloride at 3.5 ml per 100 g, followed by washing with distilled water for 2 h at a flow-rate of 1.0 ml/min to remove the weakly bound trioctylmethylammonium chloride. Emulsified turbid water came out first, then turned clear.

High-performance liquid chromatography

A Hitachi 638 high-performance liquid chromatograph was used with a UV detector at 260 nm. The column was equilibrated with an initial eluting solution and eluted with a linear gradient of sodium perchlorate solution buffered with Tris–acetate (pH 7.5) or sodium hydroxide (pH 12) containing EDTA at a flow-rate of 1.0 ml/min. The relative concentrations of sodium perchlorate, Tris buffer and EDTA were varied. Typical back-pressures of the columns were 5, 15, 35 and 120 kg/cm^2 from materials IV, III, II and I, respectively. The material from I was used in the usual operations, except in the experiment to investigate the influence of particle size on resolution.

RESULTS AND DISCUSSION

The column performance was greatly affected by the particle size of the resin, the coating method and the packing procedure. The finest materials gave the best resolution of the oligonucleotides. In most instances we used material I coated with 6 ml of trioctylmethylammonium chloride per 100 g of the resin (Ia). The retention time and resolution of the oligonucleotides changed during the first five injections partly because of the loss of the weakly bound coating from the new column. We therefore conducted a blank operation a few times with a newly packed column; the column performance became stable after this simple pretreatment. Gradients of acetate, chloride and perchlorate salt solution have previously been used as an eluent in RPC-5 column chromatography[7,13]. The column performance differs greatly with the different types of salt. We investigated solutions of sodium chloride, perchlorate and acetate as eluents and confirmed that sodium perchlorate is the most suitable. The advantages of sodium perchlorate are as follows: (1) perchlorate solution does not corrode stainless-steel HPLC equipment, whereas chloride solution is corrosive; (2) perchlorate solution has a high ionic strength and is a strong eluent[17]; sodium perchlorate solution eluted oligonucleotides at concentrations three to ten time lower than sodium chloride or acetate solution; and (3) sodium perchlorate can be removed more easily than sodium chloride from the recovered oligonucleotide solution by ethanol precipitation, as sodium perchlorate is far more soluble than sodium chloride in organic solvents. The recovered oligonucleotides after desalting retain their susceptibility to enzymes such as nucleases, polynucleotide kinase and T_4 ligase.

Fig. 1 shows a typical separation of oligoadenylates containing 5'-terminal phosphate, which was prepared by partial digestion of poly A with nuclease SW for 30 min. Oligoadenylates up to the 75-mer were successively separated in less than 2 h by HPLC on column Ia. After the enzyme digestion of the poly A for 4 h, long oligoadenylates diminished and only short-chain oligoadenylates were detected by HPLC (data not shown). The time course of the enzyme digestion of nucleic acid was monitored by HPLC.

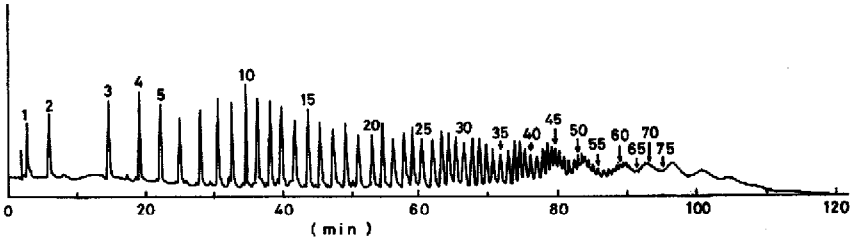

Fig. 1. Separation of oligoadenylates on an RPC-5-like column (Ia) The column (250 × 4 mm I.D.) was eluted with a gradient of $NaClO_4$ solution from 0.01 to 0.05 *M* in 30 min, then to 0.1 *M* in 50 min and finally to 0.15 *M* in 40 min, at a flow-rate of 1.0 ml/min and at 22°C. The buffer used was 10 m*M* Tris–acetate (pH 7.5) and 1 m*M* EDTA. Oligoadenylates, which were prepared by partial digestion of polyadenylates with nuclease SW for 30 min, were applied to the column without purification. The eluate was monitored by UV absorption at 260 nm. The numbers on the peaks denote the chain length of the oligoadenylate.

We investigated the effect of the particle size of the packing materials on the resolution of the oligonucleotides. Fig. 2 shows the elution profiles of the oligoadenylates when packing materials II, III and IV were used. The elution conditions were the same as in Fig. 1. The difference in particle size of the packing materials did not influence the retention time, but had a great effect on the resolution. A smaller and more uniform size of the material gave better resolution. Oligoadenylates up to the 30-mer were separated with packing material III whereas IV gave very poor resolution. Hence the material obtained by sieving between 100 and 200 or 200 and 400 mesh was found to be unsuitable for the HPLC use.

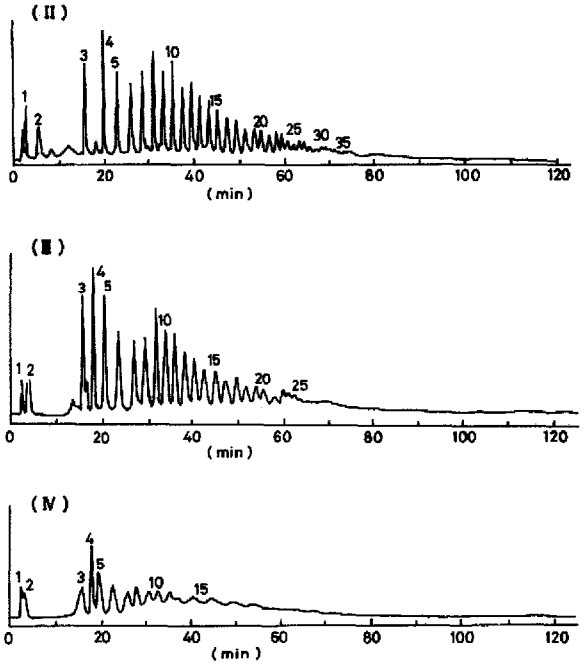

Fig. 2. Effect of particle size of RPC-5-like material on the resolution of oligoadenylates. Particle size: II, 10–40; III, 20–60; IV, 30–100 μm. Column size and elution conditions as in Fig. 1. Oligoanylates, which were prepared by partial digestion of polyadenylates with nuclease SW for 1 h, were used as samples. The numbers on the peakes denote the chain length of the oligoadenylates.

The influence of the amount of coating of trioctylmethylammonium chloride on the elution of oligoadenylates is shown in Fig. 3. Elution of the oligonucleotides was faster when a larger amount of trioctylmethylammonium chloride was used. It is interesting that the retention time decreases as the amount of stationary phase increased. Polychlorotrifluoroethylene particles have a strong hydrophobic character and bind oligonucleotides tightly[13]. Trioctylmethylammonium chloride decreases the hydrophobic binding of oligonucleotides to the polymer, although it promotes the electrostatic interactions with oligonucleotides. The packing material prepared by washing with water after coating contained a minimum amount of trioctylmethylammonium chloride, and eluted the oligonucleotides slowly. Despite previously published suggestions that RPC-5 should not be exposed to low salt concentrations or

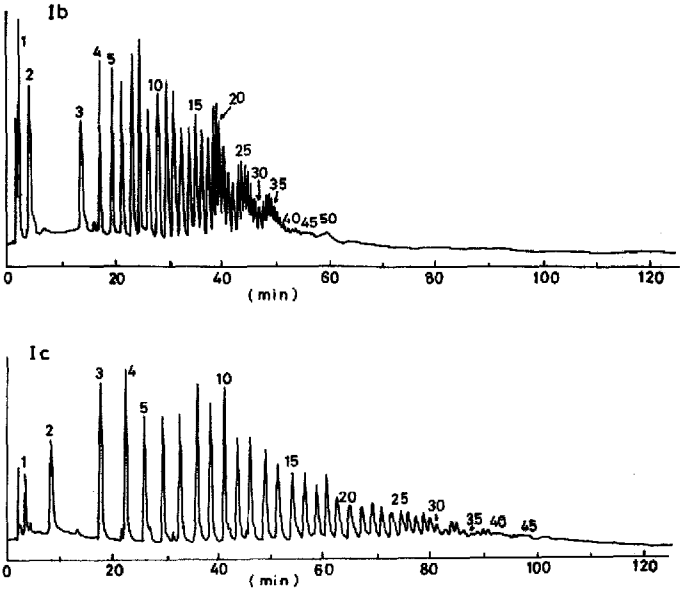

Fig. 3. Effect of amount of coating of trioctylmethylammonium chloride on RPC-5-like materials on the separation of oligoadenylates. Coating amount: Ib, 14 ml of trioctylmethylammonium chloride per 100 g of polychlorotrifuoroethylene; Ic, 3.8 ml per 100 g. Column size, elution conditions and sample as in Fig. 2.

water[7,12], we found that pretreatment of the RPC-5-like resin with water had a good effect on the separation of mononucleotides and short-chain oligonucleotides, as their retention times became longer. Adenosine, 5′-AMP, 2′-AMP, 3′-AMP, ADP and ATP were separated in less than 15 min with the prewashed column, as shown in Fig.

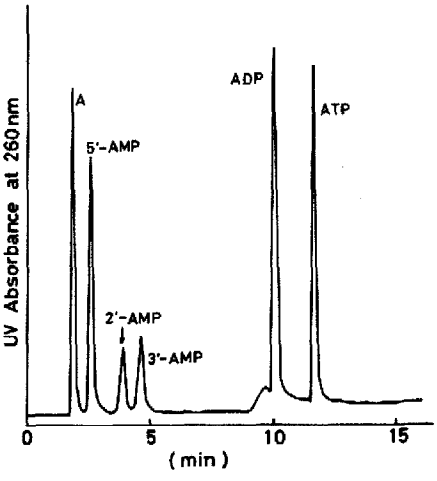

Fig. 4. Separation of adenine mononucleotides on an RPC-5-like column (Ic). The column (250 × 4 mm I.D.), pretreated with water, was eluted with a linear gradient of 0–0.01 *M* $NaClO_4$ in 15 min at a flow-rate of 1.0 ml/min and at 24°C. The buffer used was 2.5 m*M* Tris–acetate and 0.1 m*M* EDTA (pH 7.5). The eluate was monitored by UV absorption at 260 nm.

4. Compounds with more phosphate residues tend to elute later as the column has an anion-exchange character.

The separation of 2′–5′- and 3′–5′-linked isomers of oligoadenylates with the column Ic is shown in Fig. 5a. Two dimers, four trimers and four tetramers were well separated. Similarly linked isomers of oligouridylates, oligoinosinates and oligocytidylastes were resolved in a short time. Fig. 5b shows the elution profiles of the linked isomers of oligouridylates. 2′–5′-Linked isomers eluted earlier than the corresponding 3′–5′-linked isomers. Linked isomers containing both 3′–5′ and 2′–5′ linkages eluted between fully 2′–5′- and 3′–5′-linked isomers.

Four base-sequence isomeric oligodeoxyribonucleotides, GGCC, CCGG, GCGC and CGCG, showed different chromatographic patterns, as shown in Fig. 6. The mobility of the isomers was altered on changing the pH of the eluent from 7.5 to 12. The results suggest that the tetramers have different conformations which can be

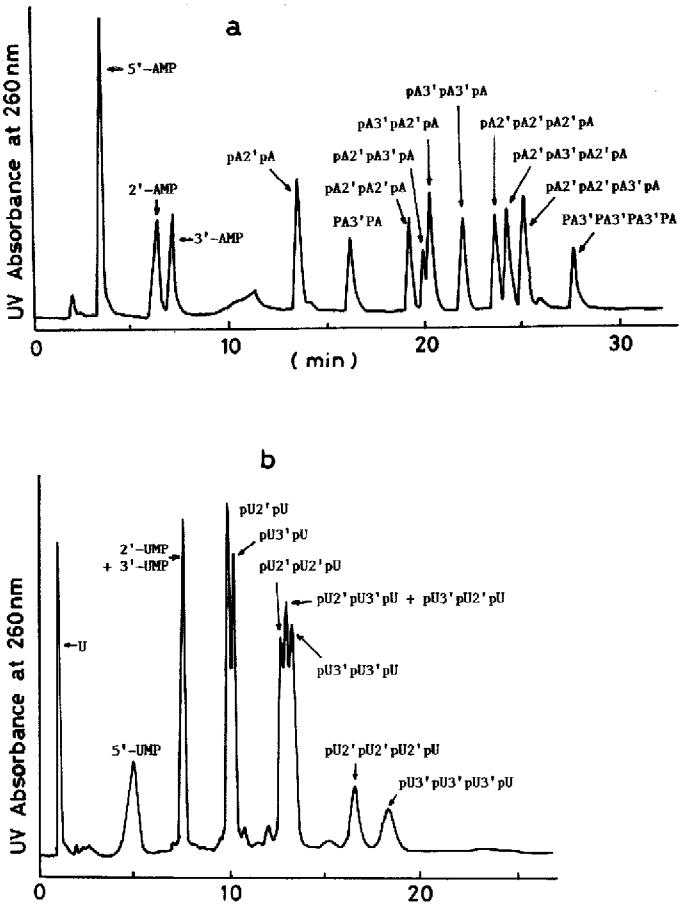

Fig. 5. Resolution of lined isomers of short-chain oligoribonucleotides. The column (Ic, 250 × 4 mm I.D.) was eluted with a linear gradient of 0–0.02 *M* $NaClO_4$ in 30 min at a flow-rate of 1.0 ml/min and at 22°C. The buffer was 2.5 m*M* Tris–acetate and 0.1 m*M* EDTA (pH 7.5). The elutate was monitored by UV absorption at 260 nm. (a) Linked isomers (2′–5′ and 3′–5′) of oligoadenylates; (b) linked isomers (2′–5′ and 3′–5′) of oligouridylates.

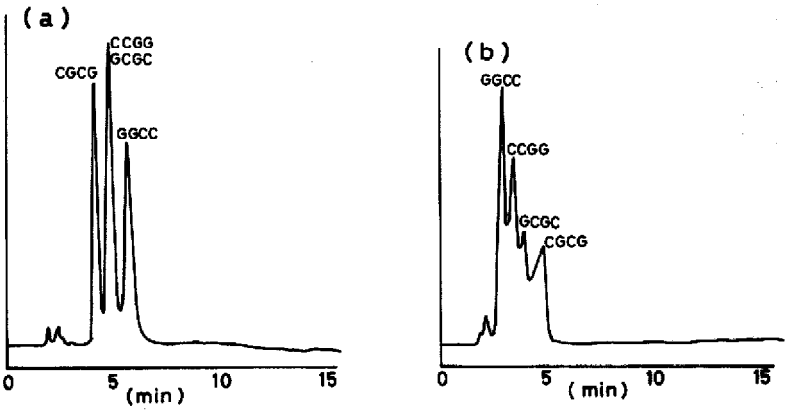

Fig. 6. Separation of sequence isomers of tetradeoxyribonucleotides, CGCG, GCGC, GGCC and CCGG, on an RPC-5-like column (Ia). The column (250 × 4 mm I.D.) was eluted with a gradient of 0.01–0.025 *M* $NaClO_4$ in 15 min at a flow-rate of 1.0 ml/min and at 22°C. Buffer (a) 10 m*M* NaOH and 1 m*M* EDTA (pH 12); (b) 10 *M* Tris–acetate and 1 m*M* EDTA (pH 7.5). The eluate was monitored by UV absorption at 260 nm.

recognized on the surface of the resin. Different elution patterns depending on the base sequence were also obversed with other sequence isomers containing AT bases, as shown in Fig. 7.

RPC-5 has generally been used for the separation of long-chain oligonucleotides such as DNA restriction fragments and t-RNA. Our results demonstrate that RPC-5 is also useful for the separation of mononucleotides and short-chain oligonucleotides. The capacity of the resin is several to ten times smaller than that of ODS-silica gel with the same column size, as the polychlorotrifluoroethylene particles are non-porous and the surface area is smaller than that of silica gel. Up to twenty A_{260} units of oligoadenylates from the dimer to decamer could be loaded on the column without a decrease in the resolution under the normal operating conditions. The

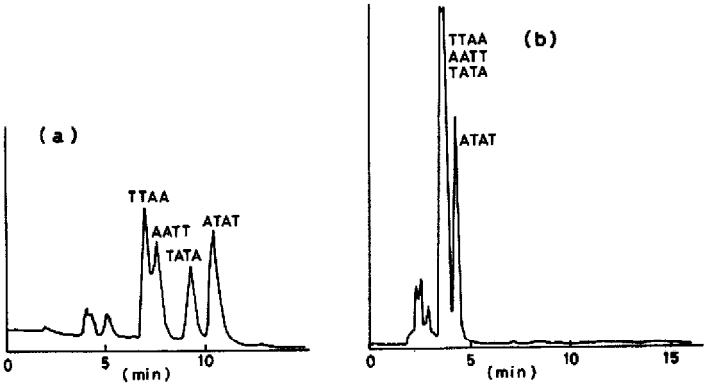

Fig. 7. Separation of sequence isomers of tetradeoxyribonucleotides, ATAT, TATA, TTAA and AATT, on an RPC-5-like column. Column and elution conditions as in Fig. 6. The minor impurities eluted in front of the tetramers.

loading capacity of the oligodeoxyribonucleotides on the resin is smaller than that of oligoribonucleotides. Up to five A_{260} units of dATAT could be chromatographed on the column with satisfactory resolution. When the samples were overloaded, the resolution became worse and the retention time became shorter.

The recoveries of the samples from the column were usually over 70%; 95% of 3′–5′-linked $(pA)_4$ was recovered when one A_{260} unit of the sample was chromatographed, as calculated from the ratio of the UV absorption of the recovered sample to that of the injected sample. The recovery of synthetic DNA oligomers, dATAT and the 25-mer, was 60–90% when 0.01 *M* sodium hydroxide solution was used as the eluting buffer. Resolution of the oligonucleotides was achieved, depending on the differences in chain length, internucleotide linkage, base sequence and phosphate residues. RPC-5 has been reported to separate DNA fragments based on chain length and base composition[7]. DNA, which is rich in AT, eluted slowly[18]. These complexities of the separation mode make the use of RPC-5-type materials difficult for identifying the size of the oligonucleotides. The separation of oligonucleotides can be accomplished based on size alone by gel electrophoresis or size exclusion chromatography with a gel filtration HPLC column[19]. On the other hand, gel electrophoresis cannot separate oligonucleotides of the same chain length but with different base compositions or different base sequences. The separation of oligonucleotides by HPLC on the RPC-5 column may complement separations by gel electrophoresis or size exclusion chromatography. A particle size of about 5 μm is most suitable in an RPC-5 HPLC column and gives rapid separations. The column can be used at least several tens of times without a decrease in resolution. The RPC-5 column has a shorter life than bonded-phase columns. Nevertheless, the RPC-5-type resin is still useful for the separation of short- and long-chain oligonucleotides, as it has a wide range of column performance depending on the operating conditions. The resolution of long-chain DNA and RNA using RPC-5-like resin will be described in a later paper.

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